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# EFFECT OF CHRONIC ETHANOL ADMINISTRATION ON DRUG METABOLISM IN ISOLATED HEPATOCYTES WITH EMPHASIS ON PARACETAMOL ACTIVATION

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**Abstract**—Chronic administration of ethanol to rats for 6–8 weeks caused an elevation in the concentration of cytochrome P-450 and an enhancement of the rates of drug oxidation and UDP-glucuronic acid conjugation in isolated hepatocytes. The oxidation of harmine was stimulated by 100 per cent with a concomitant increase in the glucuronide formation. When paracetamol and harmol were used as specific substrates of conjugation reactions, the rate of glucuronide formation was increased by about 50 per cent, while the rate of sulphate conjugation was not affected. The cytochrome P-450 dependent activation of paracetamol, measured as glutathione conjugate formation, increased about three-fold. The intracellular GSH concentration, which was not affected by ethanol treatment, decreased stoichiometrically with the formation of paracetamol–glutathione conjugate. Furthermore, the binding affinity of paracetamol to cytochrome P-450 was increased after ethanol administration. In isolated kidney cells the formation of sulfhydryl conjugates of paracetamol was increased after chronic ethanol administration.

Chronic administration of ethanol to rats has been shown to cause changes in the morphology of mitochondria and endoplasmic reticulum [1, 2] and alterations in a variety of biochemical parameters [3, 4] including increased capacity of enzymes metabolizing xenobiotics [5, 6]. This has been demonstrated both *in vitro* and *in vivo* [7–9]. However, the mechanism for these alterations is not fully understood.

Paracetamol (acetaminophen) is an analgesic and antipyretic drug which in large overdoses can cause liver necrosis in man and laboratory animals [10, 11]. Renal damage caused by this drug has also been reported [10, 12, 13]. Paracetamol is primarily metabolized in the liver to glucuronide and sulphate conjugates [14] but also undergoes a cytochrome-P-450-dependent oxidation leading to the formation of a reactive metabolite [15]. At therapeutic doses, the liver cells protect themselves from damage by trapping the reactive metabolite with reduced glutathione (GSH). At high doses of paracetamol hepatic GSH is depleted and covalent binding to cellular macromolecules can occur. This has been suggested to be the ultimate cause of cell damage [15].

During the last few years we have used isolated hepatocytes [16–19] and kidney cells [20, 21] in studies of drug metabolism and toxicity. In the present study these cells were used to investigate the effect of chronic ethanol administration on drug oxidation and conjugation, in general, and paracetamol activation in particular. The effect on paracetamol activation is of special interest, since alcoholics are known to be more susceptible to paracetamol-induced liver and kidney damage [22, 23].

## MATERIALS AND METHODS

*Treatment of animals*. Male Sprague–Dawley rats, weighing 300–350 g, were used. The animals were divided into two groups and fed commercial laboratory chow *ad lib*. (R-3, Astra-Ewos AB, Södertälje, Sweden) throughout the whole period of treatment. The experimental group received 30% ethanol (v/v) and 25% sucrose (w/v) in the drinking water whereas the control group received 25% sucrose alone [24]. These liquids were administered for 6–8 weeks until animals were used.

*Preparations and experimental procedures*. Cells from liver and kidney were isolated by collagenase perfusion as previously described [25, 20]. The yields were 2–4 × $10^8$ cells per liver and 3 × $10^7$ cells per two kidneys. Trypan blue and NADH exclusion frequencies were 90–100 and 85–90 per cent, resepectively.

Incubations were carried out at 37° with 2–3 × $10^6$ cells/ml in Krebs–Henseleit buffer, pH 7.4, supplemented with 25 mM Hepes in rotating round-bottom flasks [26] or open tubes under 93.5% $O_2$–6.5% $CO_2$ atmosphere. Metabolites from harmine and harmol were separated on t.l.c.-plates and quantitated by fluorescence [17]. Paracetamol metabolites were analysed using high performance liquid chromatography as previously described [18]. Penetration of NADH into the cells (cell viability) was measured using the lactate dehydrogenase latency test [25]. The intracellular GSH concentration was estimated by the method of Saville [27] as described earlier [25]. Concentrations of cytochrome P-450 were determined by the method of Omura and Sato [28] as modified by Jones *et al.* [29].

Table 1. Effect of chronic ethanol administration on the oxidative and conjugative metabolism of harmine and harmol in isolated hepatocytes*

| | nmoles/$10^6$ cells/min | | | | | | |
|---|---|---|---|---|---|---|---|
| | 200 $\mu$M harmine | | | | 200 $\mu$M harmol | | |
| Treatment | Glucuronide | Sulphate | Harmol | Total | Glucuronide | Sulphate | Total |
| Control | 0.08 ± 0.01 | 0.18 ± 0.02 | 0.05 ± 0.01 | 0.31 ± 0.02 | 1.10 ± 0.05 | 0.19 ± 0.03 | 1.30 ± 0.06 |
| Ethanol | 0.36 ± 0.04† | 0.19 ± 0.02 | 0.15 ± 0.02‡ | 0.70 ± 0.03† | 1.52 ± 0.15§ | 0.19 ± 0.01 | 1.70 ± 0.15§ |

* Values represent means ± S.E.M. of 5–7 different hepatocyte preparations.
† $P < 0.001$ compared to control.
‡ $P < 0.01$ compared to control.
§ $P < 0.05$ compared to control.

Collagenase was obtained from Boehringer Mannheim GmbH (Mannhein, Germany). Harmine hydrochloride was purchased from EGA-Chemie (Steinheim/Albuch, Germany) and harmol hydrochloride from Aldrich-Europe (Beerse, Belgium).

## RESULTS

*Effect of chronic ethanol administration on the metabolism of harmine and harmol.* In hepatocytes harmine undergoes a cytochrome-P-450-dependent O-dealkylation to harmol which is subsequently conjugated with sulphate and glucuronic acid [17]. This is also demonstrated in Table 1.

Chronic ethanol administration resulted in a more than two-fold increase in the O-dealkylation of harmine. The increase was observed both in free harmol (three-fold) and in harmol glucuronide (four-fold), whereas sulphate conjugation was unaffected. Since, in control cells, glucuronidation was far from saturated, the increase in glucuronide formation after chronic ethanol administration was in major part due to the increased formation of harmol.

In the following experiment, harmol was used to study only the conjugation reactions. In this case, the sulphate conjugation had reached its maximal capacity; the rate was the same as when harmine was used as substrate and was not increased further by the ethanol treatment. The rate of harmol glucuronide formation from added harmol was 14 times greater than that from harmine by control hepatocytes. This rate was further elevated (40 per cent) by chronic ethanol administration (Table 1).

Table 2. Concentrations of cytochrome P-450 and intracellular GSH in hepatocytes isolated from control and ethanol-treated rats*

| | nmoles/$10^6$ cells | |
|---|---|---|
| Treatment | Cytochrome P-450 | GSH |
| Control | 0.22 ± 0.01 | 45.4 ± 1.7 |
| Ethanol | 0.42 ± 0.01† | 41.5 ± 1.4 |

* Values represent means ± S.E.M. of 3–5 different hepatocyte preparations.
† $P < 0.001$ compared to control.

*Effect of chronic ethanol administration on the concentration of cytochrome* P-450 *and reduced glutathione (GSH).* Chronic ethanol treatment of rats was shown to increase the level of hepatic microsomal cytochrome P-450 per milligram protein by 50 per cent [30]. However, the hepatocytes isolated from ethanol-treated rats indicated a 100 per cent increase in cytochrome P-450 content per million cells (Table 2). This difference is most likely caused by the proliferation of endoplasmic reticulum known to occur upon chronic ethanol administration [5]. Such qualitative [30] and quantitative [5] changes may add up to the 100 per cent increase of this cytochrome (Table 2) and may be related to the overall increase of harmine metabolism (100 per cent) shown in Table 1.

Only a slight decrease of hepatocellular GSH level was obtained after the ethanol treatment (Table 2). This is in agreement with previous results obtained *in vivo* [31].

*Effect of chronic ethanol administration on the metabolism of paracetamol.* In isolated hepatocytes, paracetamol is primarily conjugated with glucuronic acid and sulphate. It also undergoes a cytochrome-P-450-dependent activation producing reactive metabolite(s). These are conjugated with intracellular GSH. The formation of glutathione conjugate thus reflects cytochrome-P-450-dependent activation of paracetamol [18]. Chronic ethanol administration resulted in a moderate increase of paracetamol glucuronidation (Table 3) which was comparable to that observed with harmol (Table 1). Sulphate conjugation was even slightly decreased. However, the

Table 3. Effect of chronic ethanol administration on paracetamol metabolism in isolated hepatocytes*

| | nmoles/$10^6$ cells/hr | | |
|---|---|---|---|
| Treatment | Glucuronide conjugate | Sulphate conjugate | Glutathione conjugate |
| Control | 43.4 ± 6.8 | 24.1 ± 6.5 | 5.5 ± 0.9 |
| Ethanol | 66.3 ± 3.5† | 18.1 ± 1.5 | 15.8 ± 0.4‡ |

* Values represent means ± S.E.M. of 3–4 different hepatocyte preparations. Paracetamol concentration was 2 mM.
† $P < 0.05$ compared to control.
‡ $P < 0.001$ compared to control.

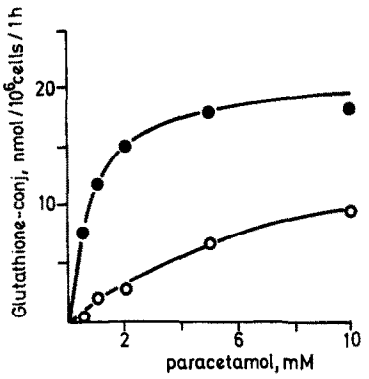


Fig. 1. Paracetamol–glutathione conjugate formation at different paracetamol concentrations in isolated hepatocytes from control (○——○) and chronic ethanol-treated rats (●——●). One experiment typical of three.

glutathione conjugate formation was increased three times (Table 3), thus reflecting an increased rate of cytochrome-P-450-dependent activation.

Such increase of paracetamol activation by ethanol treatment (three-fold) may have several underlying causes. In addition to the elevation of hepatocellular cytochrome P-450 concentration (Table 2), the affinity of paracetamol for cytochrome-P-450-dependent activation was increased considerably following ethanol treatment (Fig. 1). When the apparent $K_m$ values of paracetamol were calculated from this experiment, they were 2.8 and 0.75 mM for control and ethanol-treated rat hepatocytes, respectively.

Table 4. Formation of paracetamol–sulfhydryl conjugates at 5 mM concentration of paracetamol in kidney cells isolated from control and ethanol-treated rats*

| Treatment | nmoles/$10^6$ cells/2 hr | |
|---|---|---|
| | Cysteine | Acetylcysteine |
| Control | 0.6 | 1.0 |
| Ethanol | 1.0 | 2.3 |

* Values represent means of two different experiments.

*Effect of chronic ethanol administration on the viability of isolated hepatocytes.* Isolated hepatocytes from rat are resistant towards paracetamol-induced toxicity and maintain viability for up to 5 hr in the presence of paracetamol [18]. This is demonstrated also in Fig. 2 which describes an experiment where paracetamol glutathione conjugate formation, GSH concentrations and cell viability measured as plasma membrane integrity were followed. There was an almost direct correlation between decrease in intracellular GSH and formation of the glutathione conjugate.

Hepatocytes isolated from ethanol-treated rats showed increased rates of glutathione–paracetamol conjugate formation and of GSH depletion. However, even though the rate of activation of paracetamol was increased, these cells did not lose viability (as judged by plasma membrane integrity) for at least 3 hr.

*Effect of chronic ethanol administration on paracetamol sulfhydryl conjugate formation in isolated kidney cells.* In contrast to hepatocytes, isolated kid-

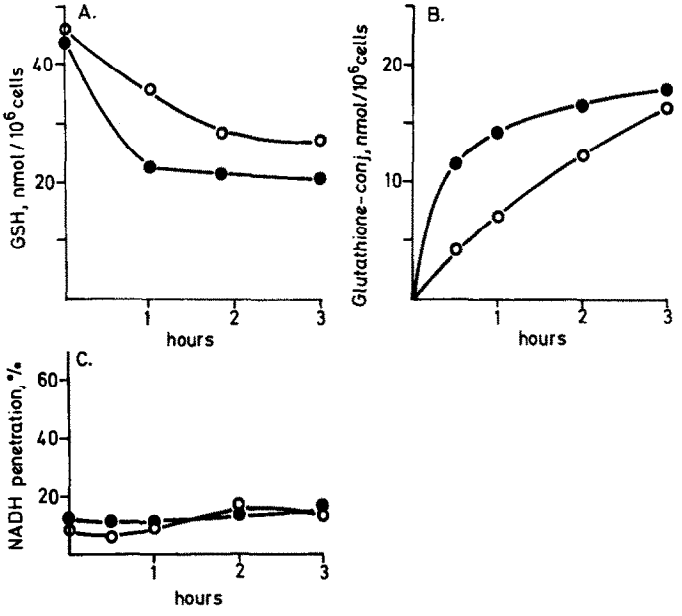


Fig. 2. Paracetamol–glutathione conjugate formation, glutathione levels and cell viability in isolated hepatocytes from control (○——○) and chronic ethanol-treated rats (●——●) in the presence of 5 mM paracetamol. Panel A, Glutathione level; Panel B, glutathione conjugate; Panel C, cell viability. One experiment typical of three.

ney cells actively catalyse the further metabolism of paracetamol-glutathione conjugate [18]. Therefore, formation of reactive metabolites from paracetamol in hepatocytes has been estimated by the level of glutathione conjugate, whereas, in kidney cells, it has to be measured by the production of cysteine and *N*-acetylcysteine conjugates due to the very rapid breakdown of preformed paracetamol–glutathione conjugate [21].

As determined by the formation of cysteine and *N*-acetylcysteine conjugates (Table 4), the rate of paracetamol activation in kidney cells is about 15 per cent of that by hepatocytes. Chronic ethanol treatment, however, increased the rates of both cysteine and *N*-acetylcysteine conjugate formation about two-fold.

## DISCUSSION

The inducing effect of chronic ethanol treatment of rats on cytochrome-P-450-dependent monooxygenation in microsomes is well documented and can, as demonstrated in this study, also be observed in isolated hepatocytes both with harmine and paracetamol as substrates. However, neither glucuronidation nor sulphate conjugation was affected to any major extent. The relatively slight increase of glucuronidation could probably, at least in part, result from the proliferation of the endoplasmic reticulum known to occur after chronic ethanol administration [5].

An increased sensitivity towards paracetamol-induced hepatotoxicity has been observed in chronic alcoholics [22, 23]. Similar results have also recently been obtained in mice which after ethanol pretreatment exhibited enhanced hepatotoxic effects of paracetamol [31]. Our observations with isolated hepatocytes suggest that the increased toxicity observed *in vivo* may be due to an increased rate of activation of paracetamol after chronic ethanol administration. That the increased toxicity of paracetamol is not due to altered intracellular GSH concentration is also indicated by our results. Ethanol will, at acute high doses, cause depletion of GSH [32] but only small changes can be observed after chronic treatment (cf. Table 2).

In addition to a quantitative change, cytochrome P-450 also seems to be qualitatively altered by ethanol treatment. There is a pronounced increase in the affinity of cytochrome P-450 for paracetamol, assuming that the cytochrome-P-450-dependent activation is the rate-limiting step in the formation of the glutathione conjugate. An enhancement in affinity could be of importance *in vivo* and contribute to an increased hepatotoxicity of paracetamol taken in therapeutic doses.

In addition to hepatotoxicity paracetamol has been reported to cause renal necrosis and failure in both man and experimental animals [10, 12, 13]. Extensive renal damage has also been observed in people who were known alcoholics and died of an overdose of paracetamol ([22], S. Orrenius, personal communication).

The renal toxicity caused by paracetamol has been suggested to result from oxidative activation of paracetamol in the kidney, since after administration of paracetamol depletion of renal GSH and covalent binding of paracetamol to renal proteins is observed [13]. Renal activation of paracetamol can also be observed in isolated rat kidney cells and is increased after chronic ethanol treatment (cf. Table 4). Whether renal activation of paracetamol is the reason for the paracetamol toxicity observed *in vivo* remains to be established. However, in view of the fact that the rate of activation in the kidney is very low compared to the liver even after ethanol treatment, this explanation seems unlikely.

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